Schreiber, David

From:	Ramirez, Delia
Sent:	Tuesday, October 07, 2003 7:48 PM
To:	Schreiber, David
Subject:	case 09/856,679

Hi David,

I would like to request the following alignment. I need seq id 2 against GI4079657.

Thank you,

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1: AAD12739. cAMP-regulated gu...[gi:4079657] BLink, Domains, Links

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DEFINITION  cAMP-regulated guanine nucleotide exchange factor I [Rattus
            norvegicus].
ACCESSION   AAD12739
VERSION     AAD12739.1  GI:4079657
DBSOURCE    locus RNU78167 accession U78167.1
KEYWORDS    .
SOURCE      Rattus norvegicus (Norway rat)
  ORGANISM  Rattus norvegicus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
            Rattus.
REFERENCE   1  (residues 1 to 884)
  AUTHORS   Kawasaki,H., Springett,G.M., Mochizuki,N., Toki,S., Nakaya,M.,
            Matsuda,M., Housman,D.E. and Graybiel,A.M.
  TITLE     A family of cAMP-binding proteins that directly activate Rap1
  JOURNAL   Science 282 (5397), 2275-2279 (1998)
  MEDLINE   99074384
   PUBMED   9856955
REFERENCE   2  (residues 1 to 884)
  AUTHORS   Kawasaki,H., Housman,D.E. and Graybiel,A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-NOV-1996) Center for Cancer Research/Department of
            Brain and Cognitive Sciences, Massachusetts Institute of
            Technology, 77 Massachusetts Avenue, E17-540, Cambridge, MA 02135,
            USA
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                     /coded_by="U78167.1:197..2851"
                     /note="GEF domain shows highest similarity to RasGEF
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      421 nkrqqilrlv srwvalyspm lrsdpvatsf lqklsdlvsr dtrlsnllre qyperrrhhr
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//
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istration and culminating in altered gene transcription, through which previous exposure to cocaine can influence the subsequent subjective qualities of the drug. Repeated exposure to cocaine causes an up-regulation of dynorphin expression through stimulation of dopamine D1-type receptors and the cAMP pathway (*2, 7, 15*). Upon subsequent exposure to cocaine, augmented release of dynorphin could inhibit local dopamine release through actions at κ opioid receptors on terminals of mesolimbic dopaminergic neurons that innervate the nucleus accumbens (*19, 20*). Diminished release of dopamine in the nucleus accumbens may be aversive, or it may unmask other actions of cocaine that oppose drug reward (*3, 21*).

With repeated use of cocaine in humans, rewarding effects of the drug reportedly diminish and are overshadowed by unpleasant side effects including anxiety and irritability (*22*). Our data provide evidence that cocaine-induced increases in CREB and dynorphin in the forebrain could contribute to these changes. Indeed, cocaine users exhibit increased expression of dynorphin mRNA in the nucleus accumbens (*23*). Up-regulation of CREB-mediated transcription in the nucleus accumbens may counteract positive feedback-type adaptations that tend to intensify drug reward [for example, see (*12, 24*)]. Sensitization to the reward-related properties of psychostimulants also contributes importantly to addictive behavior (*25*). Individual variability in the balance and time course of positive and negative feedback-type changes in brain biochemistry may ultimately influence vulnerability to addiction and relapse.

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8. Rewarding drugs establish conditioned place preferences [G. D. Carr, H. C. Fibiger, A. G. Phillips, in *The Neuropharmacological Basis of Reward*, J. M. Liebman, and S. J. Cooper, Eds. (Oxford University Press, Oxford, 1989); R. A. Wise, *ibid*]. Place conditioning was conducted with ip cocaine in a three-compartment apparatus exactly as described in (*12*).
9. Bilateral microinjections (2.0 μl) of HSV vectors were delivered over 10 min into the nucleus accumbens shell (relative to bregma: AP = +1.7, lat = ±2.3, DV = 6.8 mm below dura) or core (AP = +1.7, lat = ±3.9, DV = 6.5 mm below dura) [G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic, Sidney, 1986)] of anesthetized rats (325 to 375 g) as described in (*12*). The injection syringe was angled at 10° from the midline.
10. cDNAs for CREB and mCREB (obtained from M. E. Greenberg, Harvard University) and LacZ were inserted into the HSV amplicon HSV-PrpUC and were packaged into virus using the helper 5*dl*1.2 [R. L. Neve, J. R. Howe, S. Hong, R. G. Kalb, *Neuroscience* **79**, 435 (1997); F. Lim *et al.*, *Biotechniques* **20**, 460 (1996)]. Average titer of the purified virus stocks was 4.0×10^7 infectious units per milliliter.
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13. β-Galactosidase expression was examined as described [N. Min, T. H. Joh, K. S. Kim, C. Peng, J. H. Son, *Mol. Brain Res.* **27**, 281 (1994)]. Immunohistochemical analysis for CREB and mCREB was similar to that described in (*12*); slices were incubated with an antibody to CREB (1:1000; Upstate Biotechnology), biotinylated goat antibody to rabbit immunoglobulin G (IgG) (1:200; Vector Laboratories), and avidin-biotin (ABC Elite; Vector). The blocking solution was 1% bovine serum albumin containing 2% normal goat serum.
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17. Total RNA was isolated from bilateral punches of nucleus accumbens (Qiagen Rneasy mini kit) after unilateral gene transfer. Concentrations of dynorphin mRNA were determined by Northern blot analysis with a ^{32}P-labeled RNA probe (provided by C. R. Gerfen, National Institute of Mental Health) as described [M. Nibuya, S. Morinobu, R. S. Duman, *J. Neurosci.* **15**, 7539 (1995)].
18. norBNI (5.0 μg) was administered intracerebroventricularly (relative to bregma, AP = −0.3, lat = +1.2, DV = 4.0 mm below dura) in 2.0 μl of physiological saline over 10 min immediately before gene transfer; the drug blocks κ opioid receptors for more than 3 weeks in rats [R. Spanagel and T. S. Shippenberg, *Neurosci. Lett.* **153**, 232 (1993)].
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A Family of cAMP-Binding Proteins That Directly Activate Rap1

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cAMP (3′,5′ cyclic adenosine monophosphate) is a second messenger that in eukaryotic cells induces physiological responses ranging from growth, differentiation, and gene expression to secretion and neurotransmission. Most of these effects have been attributed to the binding of cAMP to cAMP-dependent protein kinase A (PKA). Here, a family of cAMP-binding proteins that are differentially distributed in the mammalian brain and body organs and that exhibit both cAMP-binding and guanine nucleotide exchange factor (GEF) domains is reported. These cAMP-regulated GEFs (cAMP-GEFs) bind cAMP and selectively activate the Ras superfamily guanine nucleotide binding protein Rap1A in a cAMP-dependent but PKA-independent manner. Our findings suggest the need to reformulate concepts of cAMP-mediated signaling to include direct coupling to Ras superfamily signaling.

Since the discovery that cAMP activates the phosphorylating enzyme PKA (*1*), the cAMP messenger system has been shown to involve the sequential activation (or inhibition) of cAMP production by heteromeric guanine nucleotide–binding proteins (G proteins), subsequent binding of cAMP to PKA, and consequent phosphorylation of PKA substrates (*1*). PKA is considered to be the essential effector molecule mediating many of the wide range of physiological effects initiated by receptors coupled to generation of cAMP (*1*, *2*). cAMP has also been implicated in neuronal functions, including neurotransmitter-initiated signaling and the neuroplasticity underlying development and memory (*3*, *4*), but PKA has not been clearly linked to all of these neuronal functions (*5*). We initi-

ated a search for novel brain-enriched genes related to signaling in the striatum by using a differential display protocol and by screening clones for second messenger motifs (*6*, *7*). We identified two genes characterized by the presence of cAMP-binding motifs and motifs for Ras superfamily guanine nucleotide exchange factors (GEFs), which are activators of Ras and Ras-like small G proteins (*8*). This suggested that the genes might code for cAMP-binding proteins that directly couple the cAMP signal transduction system to Ras superfamily cascades and constitute cAMP-regulated GEF proteins (cAMP-GEFI and cAMP-GEFII). We isolated *cAMP-GEFI* and *cAMP-GEFII* orthologs in humans and rats (*7*) (Fig. 1).

The cAMP-GEF proteins have similar domain structures, with a cAMP-binding domain at the NH_2 terminus, a GEF domain at the COOH terminus, and a link region in between (Fig. 1, A, D, and E). These mammalian proteins show strong structural similarity to a predicted open reading frame (T20G5.5) in *Caenorhabditis elegans* (*9*) (cel cAMP-GEF) (Fig. 1, B through E). The cAMP-binding domains of cAMP-GEF family proteins form a distinct group within the cyclic nucleotide-binding protein superfamily, with closest similarity to the B domains of PKA regulatory subunits (Fig. 1B). A PR(A or T)A motif that is present in the cAMP-binding pocket of PKA (*2*, *10*, *11*) is also conserved in the cAMP-GEF proteins (Fig. 1E). The first Ala of this motif confers specificity for cAMP as opposed to Thr, which is found in proteins that bind cyclic guanosine monophosphate (cGMP). All of the cAMP-GEF family members have Ala at this position and are therefore predicted to bind cAMP rather than cGMP (*11*).

The GEF domains of the cAMP-GEFs show high similarity to those of Ras superfamily GEF proteins but form an independent cluster distinct from Ras GEFs such as CDC25, hSos1, and rRas-GRF (Fig. 1, C and D). The three structurally conserved regions specific to Ras superfamily GEFs (*8*) are present in all of the cAMP-GEF proteins (Fig. 1D).

To identify the small G protein substrates for cAMP-GEFI and cAMP-GEFII and to determine whether their GEF activity would be altered by the binding of cAMP, we analyzed the effects of *cAMP-GEFI* and *cAMP-GEFII* expression in 293T cells on the ratio of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) bound to Ras superfamily members in the presence or absence of forskolin and 3-isobutyl-1-methylxanthine (IBMX) (Fig. 2) (*12*). In the absence of forskolin and IBMX, only Rap1 was activated (Fig. 2). In the presence of forskolin and IBMX, both cAMP-GEFI and cAMP-GEFII activated Rap1A, but not H-Ras or R-Ras, and RalA was slightly activated, by cAMP-GEFI only (Fig. 2, B and

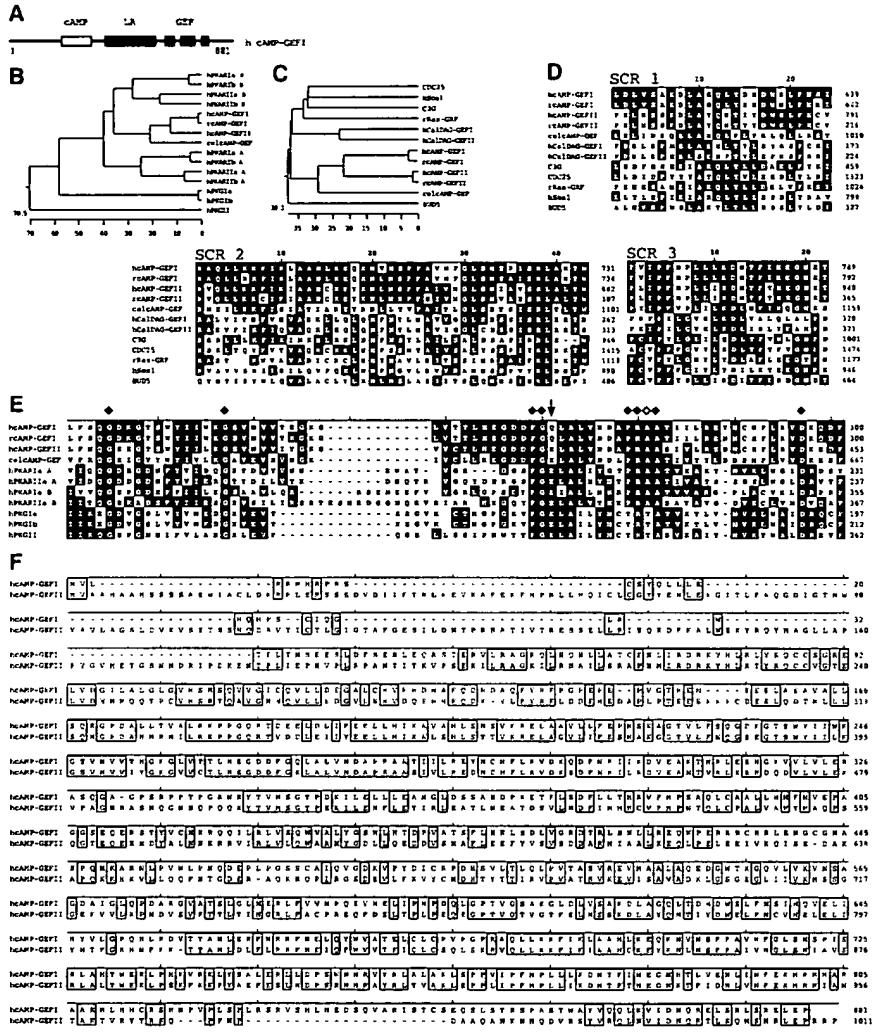

Fig. 1. Structure of cAMP-GEFs. Prefixes to protein names indicate the following: h, human; r, rat; cel, *C. elegans*. (**A**) Schematic representation of cAMP-GEF family protein motifs. LR, link region. (**B**) Phylogenetic analysis of cAMP-binding domains of cAMP-GEFI, cAMP-GEFII, and other cyclic nucleotide binding proteins. (**C**) Phylogenetic analysis of GEF domains of cAMP-GEFI, cAMP-GEFII, and other Ras superfamily GEFs. (**D**) Amino acid sequences (*10*) of the three structurally conserved regions (SCRs) of cAMP-GEFs and other Ras superfamily GEFs (black indicates identity). (**E**) Amino acid sequences of the cAMP-binding pockets of cAMP-GEFI, cAMP-GEFII, and other cyclic nucleotide–binding proteins. The positions of invariant amino acid residues are shown by black diamonds (*11*). The open diamond indicates the amino acid that determines the binding specificity for cAMP or cGMP (*11*). The arrow indicates the position of amino acid substitutions specific to cAMP-GEFs (*28*). (**F**) Full-length amino acid sequences of human cAMP-GEFI and cAMP-GEFII (boxes indicate amino acid identity) (*7*). Multiple sequence alignments and phylogenetic analyses were carried out with LASERGENE (DNASTAR, Madison, WI). Abbreviations and GenBank accession numbers of the protein sequences used here are as follows: hPKARIα (human cAMP-dependent protein kinase regulatory subunit type I-alpha), 125193; hPKARIβ, 1346362; hPKARIIα, 125198; hPKARIIβ, 400115; hPKGIα (human cGMP-dependent protein kinase type I-alpha), 1255602; hPKGIβ, 125379; hPKGII, 1906312; hCalDAG-GEFI (human calcium and diacylglycerol–regulated GEFI), U71870; hCalDAG-GEFII, AF081195; C3G, 474982; hSos1 (human son-of-sevenless 1), 476780; CDC25 (cell division control protein 25), 115914; rRas-GRF, 57665; BUD5, 171141 (*29*).

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D). The effects of forskolin and IBMX treatment on cAMP-GEFI and cAMP-GEFII were dose dependent (*12*). Treatment with forskolin and IBMX had no effect in the absence of cAMP-GEFs (Fig. 2, C and D).

We performed mutational analyses with cAMP-GEFI to examine whether its cAMP-binding domain is required for the activation of Rap1A. In contrast to wild-type cAMP-GEFI, a deletion mutant lacking a cAMP binding domain [pcDNA-rcAMP-GEFI:ΔcAMP(528) and -(595)] did not activate Rap1A with or without treatment with forskolin and IBMX (Fig. 2C) (*13*). In mutants with a single amino acid substitution in the cAMP-binding pocket known to block binding [pcDNA-rcAMP-GEFI:R(279)K] (*10*, *13*, *14*), the response to forskolin and IBMX treatment was reduced by about 30% (Fig. 2C). Thus, cAMP binding to cAMP-GEFI appears to be necessary for its cAMP-dependent activation of Rap1A.

Activation of Rap1A after the addition of forskolin and IBMX to *cAMP-GEFI* transfectants (Fig. 2E) was detected within 10 s, reached a maximum after 5 min, and continued for at least 60 min. The rapid kinetics of activation suggests a direct effect of cAMP-GEFI on Rap1A rather than secondary effects mediated by other Ras superfamily GEFs. Exposure of cells to Sp-cAMPS, an analog of cAMP, activated Rap1A to a similar extent as did treatment with forskolin and IBMX. The direct activation of Rap1 by cAMP-GEF protein was confirmed in an in vitro assay system with the purified GEF domain of cAMP-GEFII (Fig. 3E) (*15*). In vitro–translated, isotope-labeled cAMP-GEFI showed selective binding to cAMP bound to agarose beads (*16*) (Fig. 3A). Binding was inhibited by excess amounts of either cAMP or 8-Br-cAMP (Fig. 3A). Neither the deletion constructs lacking a cAMP-binding domain nor the pocket mutation construct of cAMP-GEFI showed binding activity (Fig. 3, B through D).

cAMP-dependent activation of Rap1 has been ascribed to the phosphorylation of Rap1A by PKA, which increases its binding affinity for smgGDS, a GEF with broad substrate specificity (*17*). However, in our 293T cell assay system in the absence of cAMP-GEFs, we did not detect an increase of GTP-bound Rap1A in response to increased concentrations of cAMP (Fig. 2D). Furthermore, even in the presence of H-89, a potent and selective inhibitor of PKA (*12*), cAMP-GEFI and cAMP-GEFII still activated Rap1A (Fig. 2D). These data suggest that the activation of Rap1A induced by cAMP-GEFI and cAMP-GEFII is independent of the PKA pathway.

Discrete expression patterns of human *cAMP-GEFI* and *cAMP-GEFII* were observed by Northern (RNA) analysis (*18*) (Fig. 4, A and A′). *cAMP-GEFI* was widely expressed (Fig. 4A), whereas *cAMP-GEFII* was prominent in the brain and the adrenal glands (Fig. 4A′). Both genes were expressed in some fetal tissue types for which little or no expression was detected in adult tissues (Fig. 4, C and C′). The expression patterns of the two genes in the nervous system also differed, with *cAMP-GEFI* having wider expression than *cAMP-GEFII* (Fig. 4, B and B′). These region-specific neuronal expression patterns were confirmed in in situ hy-

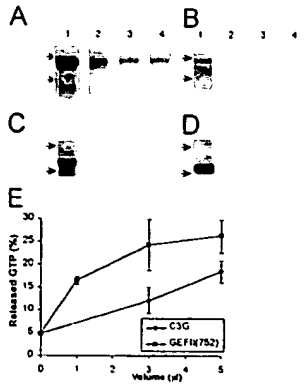

Fig. 3. Binding of in vitro–translated wild-type and mutant cAMP-GEFI proteins to cAMP coupled to agarose beads (*16*). Arrows indicate 97.4 and 68 kD in (A) and (B); 43 and 29 kD in (C) and (D). (**A**) Wild-type full-length rat cAMP-GEFI protein. (**B**) Mutant with the cAMP pocket mutation [R(279)K]. (**C** and **D**) Deletion constructs lacking the cAMP-binding domain [(C), ΔcAMP(528); (D), ΔcAMP(595)]. Lane 1, sample directly from in vitro translation; lane 2, protein bound to the beads without cAMP agonist; lane 3, same as lane 2 with 10 mM cAMP; lane 4, same as lane 2 with 10 mM 8-Br-cAMP. (**E**) Dose-dependent activation of Rap1A in vitro by purified recombinant C3G (diamonds) and the purified recombinant GEF domain of cAMP-GEFII [GEFII(752)] (squares).

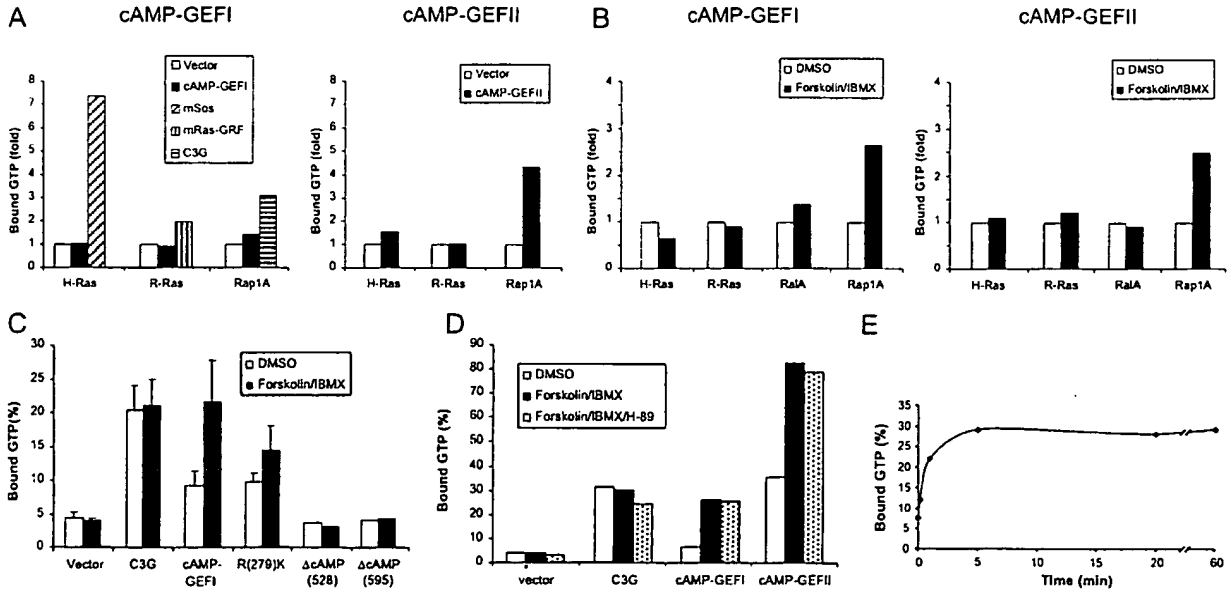

Fig. 2. cAMP-dependent activation of Rap1A by cAMP-GEF proteins (*12*). (**A**) Effects of cAMP-GEFI, cAMP-GEFII, and other Ras superfamily GEFs (mSos, mRas-GRF, and C3G) on Ras superfamily members. Fold differences were calculated by dividing each experimental value by the corresponding vector or dimethyl sulfoxide control value. (**B**) Activation of Ras superfamily members by cAMP-GEFI and cAMP-GEFII in the presence of 50 μM forskolin and 100 μM IBMX. (**C**) Mutational analysis of cAMP-GEFI showing requirement for the cAMP-binding domain (*13*). (**D**) cAMP-dependent, but PKA-independent, activation of Rap1A by cAMP-GEFI and cAMP-GEFII. (**E**) Time course of Rap1A activation of cAMP-GEFI by forskolin and IBMX.

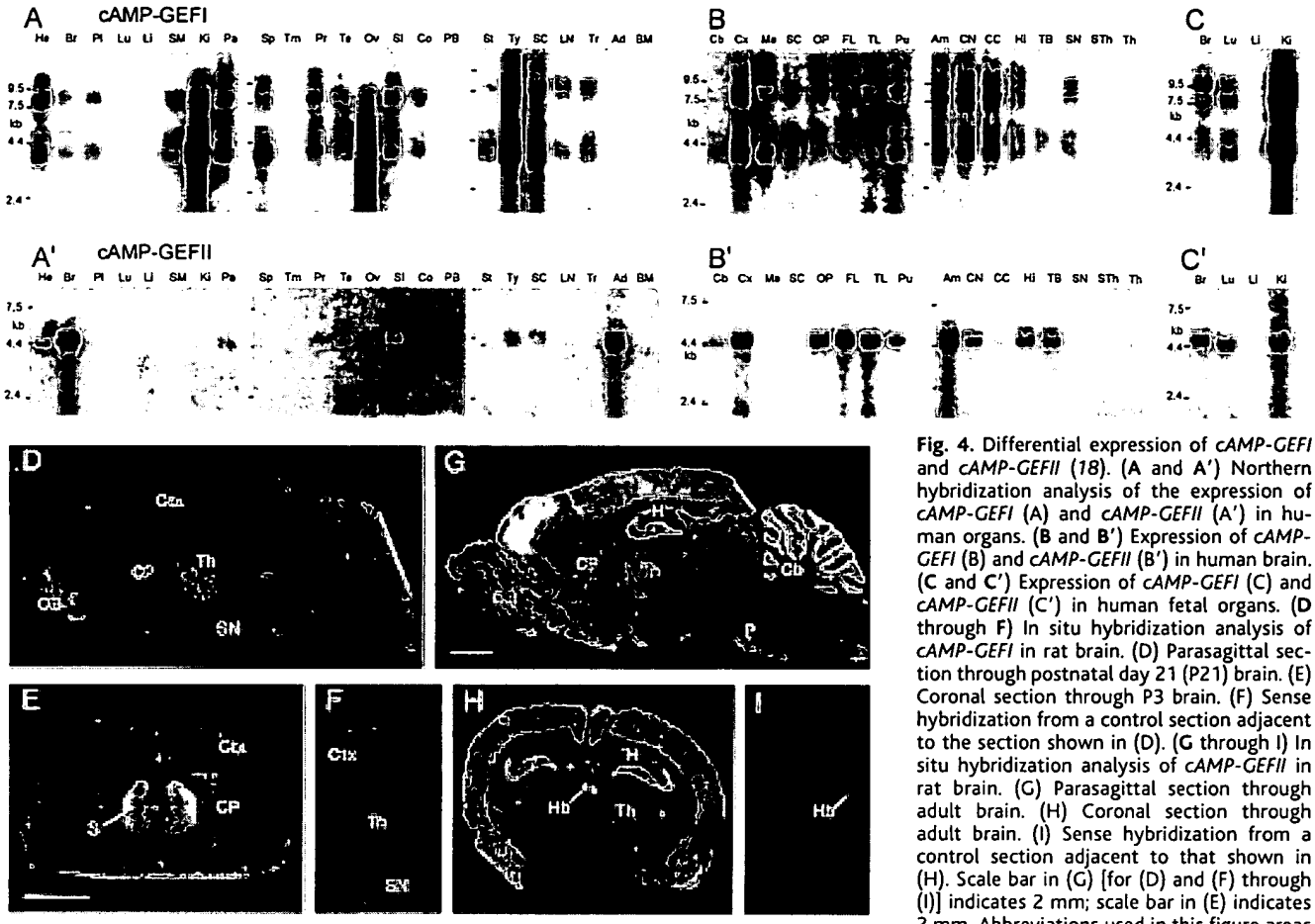

Fig. 4. Differential expression of *cAMP-GEFI* and *cAMP-GEFII* (*18*). (A and A') Northern hybridization analysis of the expression of *cAMP-GEFI* (A) and *cAMP-GEFII* (A') in human organs. (B and B') Expression of *cAMP-GEFI* (B) and *cAMP-GEFII* (B') in human brain. (C and C') Expression of *cAMP-GEFI* (C) and *cAMP-GEFII* (C') in human fetal organs. (D through F) In situ hybridization analysis of *cAMP-GEFI* in rat brain. (D) Parasagittal section through postnatal day 21 (P21) brain. (E) Coronal section through P3 brain. (F) Sense hybridization from a control section adjacent to the section shown in (D). (G through I) In situ hybridization analysis of *cAMP-GEFII* in rat brain. (G) Parasagittal section through adult brain. (H) Coronal section through adult brain. (I) Sense hybridization from a control section adjacent to that shown in (H). Scale bar in (G) [for (D) and (F) through (I)] indicates 2 mm; scale bar in (E) indicates 2 mm. Abbreviations used in this figure areas follows: Ad, adrenal gland; Am, amygdala; BM, bone marrow; Br, brain; Cb, cerebellum; CC, corpus callosum; CN, caudate nucleus; Co, colon (mucosal lining); CP, caudoputamen; Ctx, cortex; Cx, cortex; FL, frontal lobe; H, hippocampus; Hb, habenula; He, heart; Hi, hippocampus; Ki, kidney; Li, liver; LN, lymph node; Lu, lung; Me, medulla oblongata; OB, olfactory bulb; OP, occipital pole; Ov, ovary; P, pons; Pa, pancreas; PB, peripheral blood leukocytes; Pl, placenta; Pr, prostate; Pu, putamen; S, septum; SC, spinal cord; SI, small intestine; SM, skeletal muscle; SN, substantia nigra; Sp, spleen; St, stomach; Sth, subthalamic nucleus; TB, total brain; Te, testis; Th, thalamus; TL, temporal lobe; Tm, thymus; Tr, trachea; Ty, thyroid.

bridization experiments (*18*) (Fig. 4, D through I). *cAMP-GEFI* mRNA was expressed broadly at low levels in the adult brain, but it was strongly and selectively expressed in parts of the neonatal brain, including the septum and the thalamus (Fig. 4, D through F). In contrast, *cAMP-GEFII* was strongly expressed in the mature as well as the developing brain, with high mRNA levels in the cerebral cortex, the hippocampus (especially CA3 and the dentate gyrus), the habenula, and the cerebellum (Fig. 4, G through I). Genes of the *cAMP-GEF* family could have widespread influence on cAMP functions in multiple organs of the body and could contribute to region-specific functions in the brain.

Intracellular cAMP can interact directly with some ion channels (*19*), but most cAMP-mediated effects in eukaryotes have been considered as sequels to cAMP binding by the regulatory subunits of the PKA tetramer (*1*, *2*). Our data raise the possibility that some of the physiological functions of cAMP may result from direct cAMP coupling to Rap effector pathways.

cAMP can inhibit or stimulate the Ras/mitogen-activated protein (MAP) kinase pathway (*20*, *21*). The inhibition can occur at the initial translocation step by which Ras activates Raf (*20*), whereas activation of Rap1 is thought to occur through phosphorylation by PKA (*17*, *22*). Rap1, itself discovered as a negative regulator of Ras (*23*), is suspected of having independent functions as well (*20*, *23*), and activation of Rap1 has been proposed as part of a switch mechanism determining whether growth or differentiation occurs in response to nerve growth factor (*22*). Our findings suggest that different levels of *cAMP-GEF* expression could confer cell type–specific cAMP regulation of Ras superfamily signaling related to growth and differentiation.

The cAMP second messenger system has also been centrally implicated in modulating synaptic function, neuroplasticity, and cognition (*3*). Our findings demonstrating differentially high expression of the *cAMP-GEF*s in structures such as the hippocampus [implicated in memory formation (*24*)] and key limbic system structures linked to brain reward circuits and schizophrenia (*25*) suggest that the cAMP-GEFs could underlie some of these neuronal functions of cAMP.

We have identified another gene, *CalDAG-GEFI*, which codes for a protein with binding sites for calcium and diacylglycerol as well as a Rap-specific GEF (*6*). Moreover, both Ebinu *et al.* (*26*) and ourselves (*6*) have identified a second gene of the *CalDAG-GEF* family (*CalDAG-GEFII* or *RasGRP*), which links calcium and diacylglycerol inputs to a Ras-specific GEF. Thus at least three major second messenger systems are directly coupled to Ras superfamily signaling pathways by proteins that have second messenger input domains and GEF output domains. Previously, each of these second messenger systems was believed to exert its effects primarily through the activation of specific protein kinases. For cAMP-mediated

signaling, our findings suggest that direct coupling of cAMP to Rap activation by cAMP-GEFs is an important alternative cAMP messenger system.

References and Notes

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7. A 274–base pair DNA fragment encoding part of the cAMP-binding domain was isolated by a differential display method [P. Liang and A. B. Pardee, *Science* **257**, 967 (1992)] using mRNA from the striatum and forebrain of adult rats (*6*). The fragment was used as a probe to isolate full-length rat *cAMP-GEFI* cDNA from rat whole brain (λgt10 cDNA library, Clontech). Full-length cDNA of human *cAMP-GEFI* was isolated from human frontal cortex (λZAPII cDNA library, Stratagene) with rat *cAMP-GEFI* cDNA as a probe. Full-length human *cAMP-GEFII* cDNA was isolated from human frontal cortex (λZAPII cDNA library, Stratagene) with a 1.6-kb fragment derived from the expressed sequence tag (EST) clone (GenBank accession number R59283). A partial cDNA clone was isolated from rat whole brain (λZAPII cDNA library, Stratagene) with human *cAMP-GEFII* cDNA as a probe.
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10. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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12. Analysis of GTP bound to Ras superfamily G proteins was carried out as described (*6*, *27*). 293T cells were transfected with expression vectors for Ras family members tagged with glutathione S–transferase (GST) and with GEFs by the calcium phosphate method. Cells were treated with a mixture of the adenylate cyclase activator, forskolin (50 μM, Sigma) and the phosphodiesterase inhibitor IBMX (100 μM, Sigma), or with the cAMP analog Sp-cAMPS triethylamine (Sp-cAMPS) (100 μM, Research Biochemicals International) and then were lysed 5 min later (except during time-course experiments). To inhibit PKA activity, the inhibitor H-89 dihydrochloride (H-89) (10 μM, Calbiochem) was used to treat transfected cells [T. Chijiwa *et al.*, *J. Biol. Chem.* **265**, 5267 (1990)]. The dose dependency of the effects of forskolin and IBMX treatment on the activation of cAMP-GEFI and cAMP-GEFII was determined by in vivo guanine nucleotide exchange assay. Median effective concentration values of Rap1 activation by cAMP-GEFI and cAMP-GEFII were 1.8 μM and 0.3 μM, respectively. Full-length rat *cAMP-GEFI* cDNA was inserted into pcDNA3 (Invitrogen) with a COOH-terminal FLAG epitope (Kodak) to generate pcDNA-rcAMP-GEFI-FL. For the *cAMP-GEFII* construct, a fragment of human *cAMP-GEFII* amplified by polymerase chain reaction (PCR) was subcloned into a pCAGGS expression vector provided by J. Miyazaki, with the addition of a histidine (His × 6) tag at its NH_2-terminus, resulting in pCAGGS-His-hcAMP-GEFII. Constructs for Ras family members were pCAGGS-C3G and pCAGGS-mSos1 (*6*, *27*). pCAGGS-mCdc25 encoding mRas-GRF (CDC25mM) was from T. Gotoh.
13. Deletion constructs lacking a cAMP-binding domain were made from rat *cAMP-GEFI* cDNA: pcDNA-rcAMP-GEFI:ΔcAMP(528) contained amino acids 528 through 884; pcDNA-rcAMP-GEFI:ΔcAMP(595) contained amino acids 595 through 884. A mutant of rat *cAMP-GEFI* with a point mutation in the cAMP-binding pocket ([pcDNA-rcAMP-GEFI:R(279)K] was constructed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The arginine residue at amino acid 279 of rat cAMP-GEFI protein was converted to a lysine. This mutation, when made in PKA, reduces cAMP-binding activity (*14*).
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15. In vitro guanine nucleotide exchange activity assays with purified recombinant proteins were performed as described (*27*). Purified GST-tagged Rap1A was mixed with ^{32}P-α-GTP, and the mixture was incubated with unlabeled GTP and the purified GEFs, then filtered through nitrocellulose. The radioactivity of the filters was quantified after several washes. The GST-tagged expression construct of the GEF domain of human *cAMP-GEFII* [GEFII(752)] was made by inserting a PCR-amplified fragment (amino acids 752 through 1011) into pGEX-4T (Pharmacia).
16. ^{35}S-labeled, in vitro–translated cAMP-GEFI and cAMP coupled to agarose beads (Sigma) were incubated at 4°C for 1 hour in buffer containing tris-HCl (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, bovine serum albumin (1 mg/ml), and protease inhibitors. Bound proteins were separated by SDS–polyacrylamide gel electrophoresis and were detected by autoradiography. Binding was competed with 10 mM cAMP (Sigma) or with 10 mM 8-Br-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) (Sigma).
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18. Northern hybridization was done with human tissue filters from Clontech with Xho I fragments of human *cAMP-GEFI* (nucleic acids 2446 through 2974 and 2974 through 3392) and Hind III fragments of human *cAMP-GEFII* (nucleic acids 3335 through 4278) as probes. Multiple bands were observed on Northern blots of *cAMP-GEFI*. The size of the predominant band was approximately 4.0 kb, which is consistent with that of the full-length cDNA of *cAMP-GEFI*. For *cAMP-GEFII*, a single transcript 4.4 kb in size was identified. In situ hybridization was done as described (*6*). For *cAMP-GEFI*, five cDNA fragments derived from rat *cAMP-GEFI* were subcloned into pGEM-11Zf(+) (Promega) and used for riboprobe synthesis as follows: Xho I to Pst I fragment (nucleic acids 1029 through 1771), Xho I to Bbs I (1029 through 1349), Bbs I to Pst I (1349 through 1771), Pst I to Bam HI (1771 through 2118), and Xma I to Eco RI (2832 through 3373). All riboprobes were tested and gave equivalent results. For *cAMP-GEFII*, we used a construct containing the Eco RI fragment (855 through 1404) of rat *cAMP-GEFII* cDNA subcloned into pBluescriptII (Stratagene).
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28. Another invariant motif, FGE (indicated by black diamonds in Fig. 1E) (*10*), occurs 10 amino acids upstream of the PR(A/T)A motif. The FGE motif is thought to make contact with the cyclic nucleotide and to stabilize its binding to the pocket (*11*). In cAMP-GEFs, the negatively charged glutamate residue of this motif (arrow in Fig. 1E) is replaced by neutral glutamine in cAMP-GEFI and by positively charged lysine in cAMP-GEFII and in cel cAMP-GEF. A lysine substitution at this position in the human PKARIα subunit has diminished ability to bind cAMP [D. Øgreid, S. O. Doskeland, K. B. Gorman, R. A. Steinberg, *J. Biol. Chem.* **263**, 17397 (1988).] Nevertheless, cAMP-GEFII, like cAMP-GEFI, binds specifically to cAMP-bound agarose beads in vitro [H. Kawasaki *et al.*, unpublished observations] and also induces cAMP-dependent activation of Rap1A (Fig. 2).
29. The sequences reported in this paper have been deposited in the GenBank data base. Accession numbers are as follows: human cAMP-GEFI, U78168; rat cAMP-GEFI, U78167; human cAMP-GEFII, U78516; rat cAMP-GEFII, U78517.

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